

# Temporal pattern and effect of sex on lipopolysaccharide-induced stress hormone and cytokine response in pigs

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Received 18 December 2008; received in revised form 15 April 2009; accepted 17 April 2009

## Abstract

The temporal pattern and sex effect of immune and stress hormone responses to a lipopolysaccharide (LPS) challenge were assessed using a pig model. Secretion of the pro-inflammatory cytokines tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6 increased in a time-dependent manner following LPS infusion. There was also a time-dependent increase in secretion of the stress-related hormones cortisol, epinephrine (E), and norepinephrine (NE) following LPS, with peak concentrations attained within 30 min. The magnitude of the TNF- $\alpha$  and IL-1 $\beta$  responses were both positively associated ( $P < 0.05$ ) with the magnitude of cortisol response following LPS, whereas serum IL-1 $\beta$  and IL-6 were positively correlated with the magnitude of E and NE responses following LPS. Acute-phase protein production was also time-dependently increased following LPS. The concentration of immune cells in circulation was decreased ( $P < 0.05$ ) at 5.5 h post-LPS and negatively correlated with pro-inflammatory cytokine production. By 24 h post-LPS, immune cell counts increased ( $P < 0.05$ ) and were positively associated with both pro-inflammatory cytokine and stress hormone production. The amplitude of pro-inflammatory cytokine response following LPS was affected ( $P < 0.05$ ) by sex classification; however, the magnitude of elevated cytokine concentrations was not. The magnitude of the NE response, but not of the E and cortisol responses, to LPS was influenced by sex ( $P < 0.05$ ). Similar to the pro-inflammatory cytokines, the magnitude of exposure to the stress hormones following LPS was not influenced by sex. The production of serum amyloid A (SAA) was influenced by sex, with barrows producing more SAA than gilts at 24 h post-LPS ( $P < 0.05$ ). Collectively, these results demonstrate sex-specific, concomitant temporal changes in innate immune- and stress-related hormones.

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**Keywords:** Cytokine; Sex; Lipopolysaccharide; Pig; Stress

## 1. Introduction

The type of response and degree of a host's response to pathogens can markedly influence the extent of the associated morbidity, which significantly influences animal

health and subsequent economic losses in food-animal production systems [1]. The pro-inflammatory cytokines released during the innate immune system response to gram-negative bacteria such as *E. coli* directly activate the hypothalamic-pituitary-adrenal (HPA) axis, culminating in the production of cortisol from the adrenal cortex [2,3]. Cortisol serves as a negative feedback on further production of pro-inflammatory cytokines to prevent a hyperinflammatory state, which can be detrimental to the growth and health of young animals [4].

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In addition to its anti-inflammatory, immunosuppressive actions, cortisol stimulates muscle protein degradation, which compromises growth efficiency. Inflammatory cytokines can also have metabolic actions that compromise efficiency of animal growth due to their ability to stimulate skeletal muscle proteolysis and adipose tissue lipolysis [5]. Stress-induced activation of the HPA axis also initiates sympathetic nervous system (SNS) production of the catecholamines epinephrine (E) and norepinephrine (NE) [6]. However, little is known about the interactions between the SNS and immune response following an immune challenge. Therefore, the objectives of this study were to establish the temporal pattern of catecholamine response following immune activation by lipopolysaccharide (LPS) challenge in pigs, and to assess the relationship between the catecholamine, cortisol and immune responses following LPS.

## 2. Materials and methods

### 2.1. Animals, experimental design and immune challenge

All experimental procedures were in accordance with the Guide for the Care and Use of Agriculture Animals in Agricultural Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University–Kingsville. Twenty-eight cross-bred pigs were obtained from the Texas Tech University Swine Farm ( $n = 2$  barrows and 2 gilts from each of 7 litters) upon weaning at d 21 to d 28 of age. The pigs were transferred to the USDA Livestock Issues Research Unit nursery building, where they were weighed, assigned to individual pens (4 ft  $\times$  2 ft), and allowed ad libitum access to food and water. The pigs were given 2 wk to adjust to their surroundings and diet.

One day prior to LPS infusion, all 28 pigs were nonsurgically fitted with an indwelling jugular catheter according to the method of Carroll et al. [7]. Pigs were then given 24 h to recover from the cannulation procedure before collection of blood samples commenced. Prior to collection of the initial sample, an extension was attached to the catheter to allow for remote sample collection without handling the pigs. Blood samples were taken at 30-min intervals for 1 h prior to LPS infusion to establish baseline controls within each animal, as previously validated [8,9].

Lipopolysaccharide was administered manually through the catheter using a 3-mL syringe. Samples were then taken at 30-min intervals for 6 h post-LPS infusion (*Escherichia coli* 0111:B4; Sigma L-2630, Sigma Chemical, St. Louis, MO; 25  $\mu$ g/kg body weight), with

the exception of the 15-min intervals between 0 h and 1 h post-LPS for analysis of catecholamines and a 24-h sample for analysis of acute-phase proteins (APP). Approximately 5 mL of blood was drawn at each time point into a serum tube and allowed to clot for 1 h at room temperature. Tubes were then centrifuged ( $1400 \times g$  for 20 min at 20 °C), and the serum was collected, aliquoted into microcentrifuge tubes, and stored at  $-80$  °C for later analysis. Total white blood cell and white blood cell differential counts were performed on whole blood samples taken at 0, 5.5, and 24 h using a Cell-Dyn (Abbott Laboratories; Abbott Park, IL) within 1 h of collection.

### 2.2. Serum analysis

Serum concentration of cortisol (ng/mL) was determined by RIA (Coat-a-Count Assay, Diagnostic Products Corp.; Los Angeles, CA), as previously performed and validated [10]. Serum concentrations of E and NE (pg/mL) were determined using an EIA (Tri-Cat-EIA; American Laboratory Products Company, Windham, NH). Concentrations of serum cytokines were determined by ELISA (porcine TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 assays, R&D Systems, Minneapolis, MN). The serum concentrations of the APPs serum amyloid A, C-reactive protein, and haptoglobin were determined by ELISA (Tridelta PHASE RANGE Serum Amyloid A, C-reactive protein, and Haptoglobin assays; Tridelta Diagnostic Products Inc., Morris Plains, NJ). All assays were performed in duplicate, and intra- and interassay coefficient of variance (CV) values were calculated. The intra- and interassay coefficients of variation (CV) were 6% and 8%, respectively, for cytokine analyses and 10% and 12%, respectively, for the catecholamines. For APPs, the intra- and interassay CV were less than 6% and 8%, respectively.

### 2.3. Statistical analysis

Two pigs (1 barrow and 1 gilt) died as a result of LPS infusion, and data obtained from these animals were excluded from the analyses. All data from surviving pigs ( $n = 26$ ) were subjected to analysis of variance specific for repeated measures using the mixed procedure of SAS (SAS Inst., INC., Cary, NC, USA). Sources of variation included litter, time, sex, and their interactions. Specific treatment comparisons were made using Fisher's protected least significant difference, with comparisons of  $P < 0.05$  considered significant. Calculations for area under the curve (AUC) were determined using the trapezoid method of SigmaPlot (Systat Software, Inc., San Jose, CA, USA). Pearson's correlation coef-

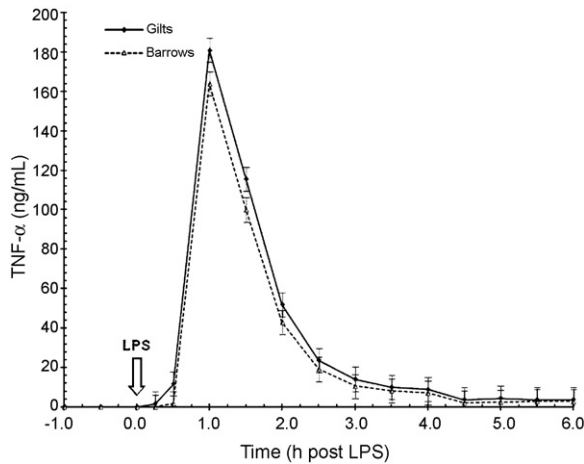


Fig. 1. Serum concentration of TNF- $\alpha$  following an i.v. challenge with lipopolysaccharide (LPS; 25  $\mu$ g/kg). Blood samples were taken for 1 h prior to and 6 h following LPS infusion. Points represent the mean  $\pm$  standard error of the mean of  $n = 13$  barrows and  $n = 13$  gilts. Lipopolysaccharide induced a time-dependent increase ( $P < 0.01$ ) in TNF- $\alpha$  following LPS. Gilts had a larger ( $P < 0.05$ ) amplitude of response following LPS than barrows. There was no sex-by-time interaction ( $P = 0.96$ ).

ficients were determined among either the amplitude of responses (peak concentrations) or among the magnitude of the responses (AUC). In almost all cases, the strongest relationships were found when analyses were conducted among the magnitudes of the response, and these coefficients are presented.

### 3. Results

#### 3.1. Pro-inflammatory cytokines

Serum TNF- $\alpha$  concentration prior to LPS (–1 to 0 h) was low, with gilts having a slightly higher ( $P < 0.01$ ) basal concentration than barrows ( $0.3 \pm 0.03$  vs  $0.2 \pm 0.03$  ng/mL, respectively). As expected, LPS induced a time-dependent increase ( $P < 0.01$ ) in TNF- $\alpha$ , with an initial increase ( $P < 0.01$ ) apparent by approximately 0.5 h post-LPS, and peak concentration occurred at 1 h post-LPS (Fig. 1). Serum TNF- $\alpha$  then rapidly decreased ( $P < 0.01$ ), reaching baseline concentration (ie, pre-LPS) by 4.5 h post-LPS. Peak concentration of TNF- $\alpha$  was influenced by sex, with gilts having a higher ( $P < 0.05$ ) peak concentration than barrows ( $180 \pm 5$  vs  $163 \pm 5$  ng/mL, respectively). However, the temporal response of TNF- $\alpha$  production following LPS was similar in both sexes ( $P = 0.96$ ), and sex did not affect ( $P = 0.18$ ) the magnitude of exposure to TNF- $\alpha$  as measured by AUC ( $212 \pm 17$  and  $177 \pm 17$  for gilts and barrows, respectively).

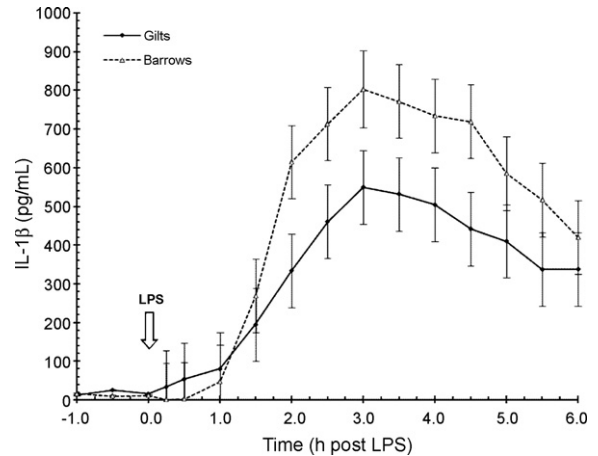


Fig. 2. Serum concentration of IL-1 $\beta$  following an i.v. challenge with lipopolysaccharide (LPS; 25  $\mu$ g/kg). Blood samples were taken for 1 h prior to and 6 h following LPS infusion. Points represent the mean  $\pm$  standard error of the mean of  $n = 13$  barrows and  $n = 13$  gilts. Lipopolysaccharide induced a time-dependent increase ( $P < 0.01$ ) in serum IL-1 $\beta$ . Barrows had a larger ( $P < 0.01$ ) amplitude of response to LPS than gilts. There was no sex-by-time interaction ( $P = 0.48$ ).

Similar to TNF- $\alpha$ , serum IL-1 $\beta$  concentration prior to LPS (–1 to 0 h) was low, with gilts having a slightly higher ( $P < 0.01$ ) basal concentration than barrows ( $17 \pm 1$  vs  $12 \pm 1$  pg/mL, respectively). Lipopolysaccharide induced a time-dependent increase ( $P < 0.01$ ) in IL-1 $\beta$ , with an increase ( $P < 0.01$ ) in serum IL-1 $\beta$  apparent by 1.5 h post-LPS and a peak concentration occurring at 3 h post-LPS (Fig. 2). Serum IL-1 $\beta$  concentration then steadily decreased ( $P < 0.01$ ), but unlike TNF- $\alpha$ , it remained elevated above baseline ( $P < 0.01$ ) throughout the 6-h post-LPS sampling period. Peak concentration of IL-1 $\beta$  was influenced by sex, with barrows having a greater ( $P < 0.01$ ) amplitude of IL-1 $\beta$  response following LPS infusion than gilts ( $802 \pm 95$  vs.  $549 \pm 90$  pg/mL, respectively). However, the duration of IL-1 $\beta$  production following LPS was not influenced by sex ( $P = 0.46$ ), and the magnitude of exposure to IL-1 $\beta$  was not different ( $P = 0.29$ ) between gilts and barrows (AUC =  $1684 \pm 480$  and  $2420 \pm 480$ , respectively).

In contrast to TNF- $\alpha$  and IL-1 $\beta$ , IL-6 was not detectable (ie, mean concentration did not differ from zero;  $P = 0.80$ ) in either barrows or gilts prior to LPS (–1 to 0 h). Lipopolysaccharide did induce a time-dependent increase ( $P < 0.01$ ) in serum IL-6, with an initial increase apparent by approximately 1 h post-LPS (Fig. 3), and a peak concentration occurred at 2.5 h post-LPS. In contrast to the temporal patterns of TNF- $\alpha$  and IL-1 $\beta$ , serum IL-6 demonstrated a biphasic pattern of production following LPS, with a secondary peak occurring at 3.5 h post-LPS. Serum IL-6 then

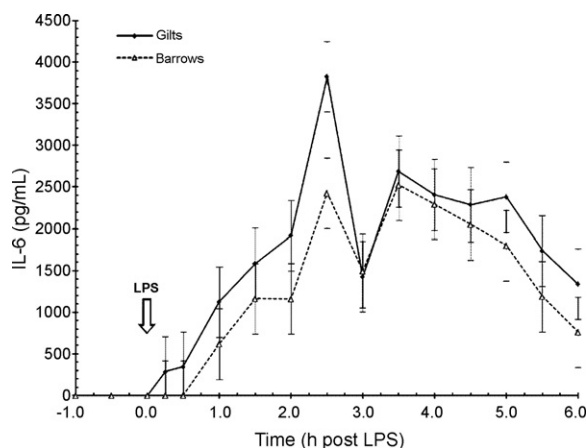


Fig. 3. Serum concentration of IL-6 following an i.v. challenge with lipopolysaccharide (LPS; 25  $\mu$ g/kg). Blood samples were taken for 1 h prior to and 6 h following LPS infusion. Points represent the mean  $\pm$  standard error of the mean of  $n=13$  barrows and  $n=13$  gilts. Lipopolysaccharide induced a time-dependent increase ( $P<0.01$ ) in serum IL-6. Gilts had a larger ( $P<0.01$ ) amplitude of response to LPS than barrows. There was no sex-by-time interaction ( $P=0.99$ ).

decreased ( $P<0.01$ ), but similar to the pattern for IL-1 $\beta$ , serum IL-6 remained elevated above baseline ( $P<0.05$ ) throughout the post-LPS period. Peak concentration of IL-6 was influenced by sex, with gilts having a greater ( $P<0.01$ ) amplitude of response than barrows ( $3827 \pm 423$  vs  $2464 \pm 423$  pg/mL, respectively). However, as with TNF- $\alpha$  and IL-1 $\beta$ , sex affected neither the temporal pattern of IL-6 ( $P=0.98$ ) nor the magnitude of exposure to IL-6 following LPS (AUC =  $8183 \pm 1656$  and  $7195 \pm 1656$  for gilts and barrows, respectively).

Correlation analysis pooled by sex indicated a positive relationship between the amplitude of the IL-6 and IL-1 $\beta$  response ( $r=0.37$ ,  $P<0.05$ ) and the IL-6 and TNF- $\alpha$  response following LPS ( $r=0.60$ ,  $P<0.01$ ). In contrast, there was no significant relationship between the amplitude of the IL-1 $\beta$  and TNF- $\alpha$  responses following LPS ( $r=0.17$ ,  $P=0.39$ ). Correlation analysis also indicated positive relationships between the magnitude of exposure to IL-6 and IL-1 $\beta$  ( $r=0.78$ ,  $P<0.01$ ) and IL-6 and TNF- $\alpha$  following LPS ( $r=0.62$ ,  $P<0.01$ ). In addition, there was a tendency for a positive relationship between the magnitude of exposure to IL-1 $\beta$  and TNF- $\alpha$  ( $r=0.35$ ,  $P=0.07$ ).

### 3.2. Stress hormones

Prior to LPS infusion, baseline concentrations of the stress hormones cortisol, E, and NE averaged  $8.07 \pm 1$  ng/mL,  $71 \pm 8$  pg/mL, and  $1293 \pm 93$  pg/mL, respectively, across male and female pigs. Lipopolysac-

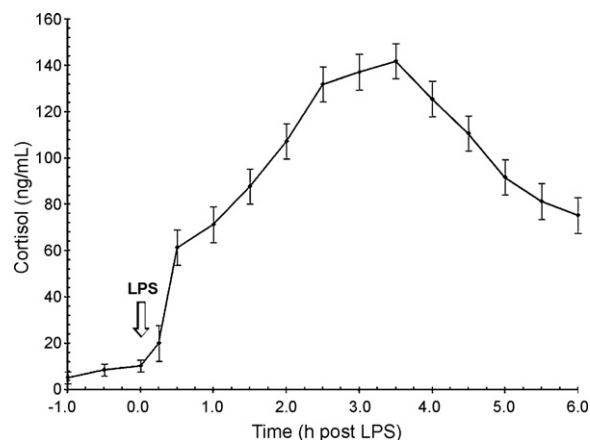


Fig. 4. Serum concentration of cortisol following an i.v. challenge with lipopolysaccharide (LPS; 25  $\mu$ g/kg). Blood samples were taken for 1 h prior to and 6 h following LPS infusion. Points represent the mean  $\pm$  standard error of the mean ( $n=26$ ). Lipopolysaccharide induced a time-dependent increase ( $P<0.01$ ) in serum cortisol. Sex did not influence ( $P=0.16$ ) the cortisol response following LPS.

charide activated both the HPA axis and the SNS components of stress response. An increase in serum cortisol was apparent by approximately 0.5 h post-LPS infusion (Fig. 4), with peak concentration occurring at 3.5 h post-LPS. Serum cortisol then began to decrease, but it remained above baseline throughout the 6-h post-LPS sampling period. Neither peak concentration of cortisol nor the temporal pattern of the cortisol response was affected by sex ( $P=0.16$  and  $0.96$ , respectively). Furthermore, the magnitude of cortisol response post-LPS was also not different ( $P=0.70$ ) between both sexes (AUC =  $513 \pm 47$  and  $487 \pm 47$  for barrows and gilts, respectively). Activation of the SNS branch of the stress response was apparent as reflected by the rapid increases ( $P<0.01$ ) in serum NE and E by 0.25 h post-LPS infusion. Serum NE concentrations peaked by 0.25 h post-LPS and began to rapidly decrease, reaching approximate baseline values by 1.5 h post-LPS (Fig. 5). Neither the peak NE concentration nor the temporal pattern of the NE response to LPS was affected by sex ( $P=0.83$ ). However, the overall serum NE concentration was influenced by sex ( $P<0.01$ ), with barrows having a consistently higher concentration of NE throughout the sampling period ( $2258 \pm 88$  vs  $1472 \pm 88$  pg/mL for barrows and gilts, respectively). Furthermore, there was a tendency ( $P=0.08$ ) for the magnitude of the NE response to be affected by sex (AUC =  $10,893 \pm 1665$  vs  $6430 \pm 1665$  for barrows vs gilts, respectively).

Similar to the NE response, serum E also increased rapidly following LPS infusion, with peak concentration occurring at 0.5 h post-LPS (Fig. 6). In contrast to

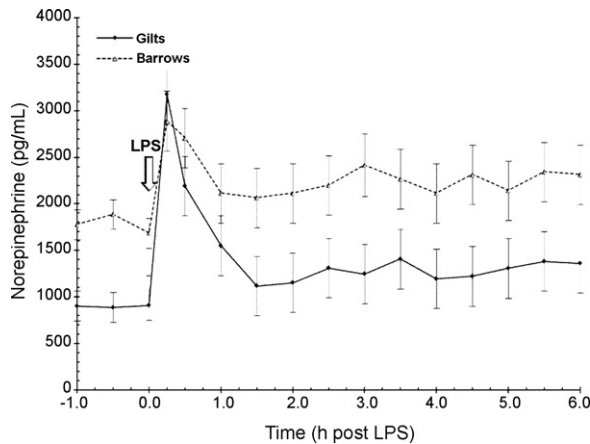


Fig. 5. Serum concentration of norepinephrine following an i.v. challenge with lipopolysaccharide (LPS; 25  $\mu$ g/kg). Blood samples were taken for 1 h prior to and 6 h following LPS infusion. Points represent the mean  $\pm$  standard error of the mean of  $n=13$  barrows and  $n=13$  gilts. Lipopolysaccharide induced a time-dependent increase ( $P<0.01$ ) in serum norepinephrine. Barrows had a greater ( $P<0.01$ ) concentration of norepinephrine than gilts. There was no sex-by-time interaction ( $P=0.83$ ).

the rapid decrease in NE concentration following peak, E concentrations did not reach approximate baseline concentrations until 3.5 to 4 h post-LPS. The E concentration then remained numerically elevated for the remainder of the sampling period, but the values were not different ( $P>0.05$ ) than the 0 h sample. Neither the temporal pattern nor the magnitude of E response following LPS infusion was affected by sex ( $P=0.35$  and  $0.67$ , respectively). Furthermore, the magnitude of the E response did

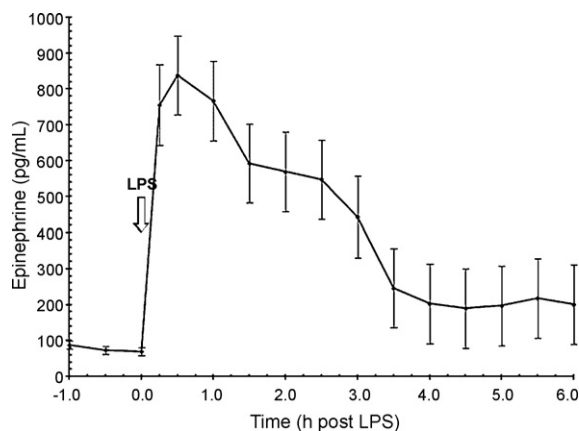


Fig. 6. Serum concentration of epinephrine following an i.v. challenge with lipopolysaccharide (LPS; 25  $\mu$ g/kg). Blood samples were taken for 1 h prior to and 6 h following LPS infusion. Points represent the mean  $\pm$  standard error of the mean ( $n=26$ ). Lipopolysaccharide induced a time-dependent increase ( $P<0.01$ ) in serum epinephrine. Sex did not influence ( $P=0.23$ ) the epinephrine response following LPS.

not differ between barrows and gilts ( $AUC = 2167 \pm 370$  and  $2395 \pm 370$ , respectively).

Correlation analysis indicated a tendency for a positive relationship between the amplitude of the NE and E response following LPS ( $r=0.37$ ,  $P=0.06$ ). In contrast, there was no relationship between the amplitude of the NE and cortisol response ( $r=0.16$ ;  $P=0.43$ ), nor the E and cortisol response following LPS infusion ( $r=0.22$ ;  $P=0.27$ ). A strong positive relationship between the magnitude of the NE and E responses was apparent when AUC was analyzed ( $r=0.54$ ,  $P<0.01$ ). However, the lack of significant relationships between NE and cortisol, and E and cortisol remained consistent.

With the exception of a positive relationship between the amplitude (peak) of the TNF- $\alpha$  and cortisol responses to LPS ( $r=0.39$ ,  $P<0.05$ ), there were no significant relationships found between the amplitudes of any of the cytokines and the amplitude of the stress hormone responses following LPS. However, the magnitudes of the cytokine responses and stress hormones were related, with positive relationships found between the magnitude of the TNF- $\alpha$  and cortisol responses ( $r=0.39$ ;  $P<0.05$ ), and with the magnitude of the IL-1 $\beta$  and all 3 stress hormones measured ( $r=0.40$ ,  $0.56$ , and  $0.56$  for cortisol, E, and NE, respectively;  $P<0.01$ ). In contrast, the magnitude of the IL-6 response was strongly related ( $P<0.05$ ) to the magnitude of both E and NE ( $r=0.54$  and  $0.44$ , respectively), but it was unrelated ( $P=0.22$ ) to the magnitude of the cortisol response.

### 3.3. Leukocyte count

The mean WBC count prior to LPS infusion (0 h) was  $11.77 \pm 0.62 \times 10^3$  cells/ $\mu$ L, and it was not different ( $P=0.76$ ) between barrows and gilts. As would be expected in normal pigs, lymphocytes and neutrophils accounted for  $>90\%$  of the total WBC present in the peripheral circulation, with monocytes (6.7%), eosinophils (1.4%), and basophils (1.0%) accounting for  $<10\%$  of the total WBC count (Table 1). Lipopolysaccharide challenge of pigs resulted in an initial redistribution of leukocytes out of the blood into the tissues, followed by proliferation-induced increases in leukocyte numbers. The initial redistribution of leukocytes in response to LPS was reflected in an approximate 45% decrease ( $P<0.01$ ) in WBC by 5.5 h post-LPS ( $11.77 \pm 0.62$  vs  $5.29 \pm 0.05 \times 10^3$  cells/ $\mu$ L at 0 vs 5.5 h, respectively) and was not affected by sex ( $P>0.05$ ). This redistribution was composed primarily of lymphocytes, which decreased by 87% ( $5.85 \pm 0.24$  vs  $0.75 \pm 0.24 \times 10^3$  cells/ $\mu$ L at 0 vs 5.5 h, respectively), and to a quantitatively lesser extent by monocytes,

Table 1  
Effect of LPS challenge on WBC counts.

Item ( $\times 10^3/\mu\text{L}$ )	Time (h post-LPS)			SEM
	0	5.5	24	
WBC	11.77 <sup>a</sup>	5.29 <sup>b</sup>	18.22 <sup>c</sup>	0.62
Monocytes	0.79 <sup>a</sup>	0.18 <sup>b</sup>	1.06 <sup>c</sup>	0.05
Lymphocytes	5.85 <sup>a</sup>	0.75 <sup>b</sup>	6.90 <sup>c</sup>	0.24
Neutrophils	4.95 <sup>a</sup>	4.32 <sup>a</sup>	9.85 <sup>b</sup>	0.69
Basophils	0.12 <sup>a</sup>	0.03 <sup>b</sup>	0.26 <sup>c</sup>	0.02
Eosinophils	0.17 <sup>a</sup>	0.01 <sup>b</sup>	0.37 <sup>c</sup>	0.02

Abbreviations: LPS lipopolysaccharides; SEM standard error of the mean; WBC white blood cells.

<sup>a,b,c</sup> Values within a row with different superscripts are different ( $P < 0.01$ ).

basophils, and eosinophils, which decreased by 77%, 75%, and 94%, respectively. In contrast, neutrophil concentration remained virtually unchanged ( $P > 0.05$ ) from 0 to 5.5 h ( $4.95 \pm 0.69$  vs  $4.32 \pm 0.69 \times 10^3$  cells/ $\mu\text{L}$ , respectively).

There were strong negative relationships between the magnitude of the TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 responses and lymphocyte numbers at 5.5 h post-LPS ( $r = -0.45$ ,  $-0.42$ , and  $-0.48$ , respectively;  $P < 0.05$ ). There were also negative relationships found between the magnitude of the TNF- $\alpha$  and IL-6 response and monocyte numbers at 5.5 h post-LPS ( $-0.39$  and  $-0.35$ , respectively;  $P < 0.05$ ). The magnitudes of the TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 responses were not related ( $P > 0.30$ ) to the number of neutrophils, eosinophils, or basophils at 5.5 h post-LPS. In contrast to the pro-inflammatory cytokines, the only significant negative relationships identified between the magnitude of the stress hormone responses (AUC) and leukocyte counts at 5.5 h were between NE and the number of lymphocytes ( $r = -0.42$ ;  $P < 0.05$ ) and E and the number of basophils ( $r = -0.40$ ;  $P < 0.05$ ). In addition, there was a positive relationship identified between the magnitude of the cortisol response and neutrophil numbers ( $r = 0.39$ ;  $P < 0.05$ ).

### 3.4. Acute-phase proteins

The APPs C-reactive protein (CRP), serum amyloid A (SAA), and haptoglobin were all detectable at relatively low concentrations prior to LPS challenge at 0 h ( $30 \pm 3.41 \mu\text{g/mL}$ ,  $4.67 \pm 34 \mu\text{g/mL}$ , and  $0.96 \pm \text{mg/mL}$ , respectively), and the basal concentrations of these proteins were not affected by sex classification ( $P > 0.05$ ). The increase ( $P < 0.01$ ) in CRP concentration was apparent by 6 h post-LPS (data not shown) and continued to increase ( $P < 0.01$ ) from 6 to 24 h post-infusion. Serum concentration of CRP was

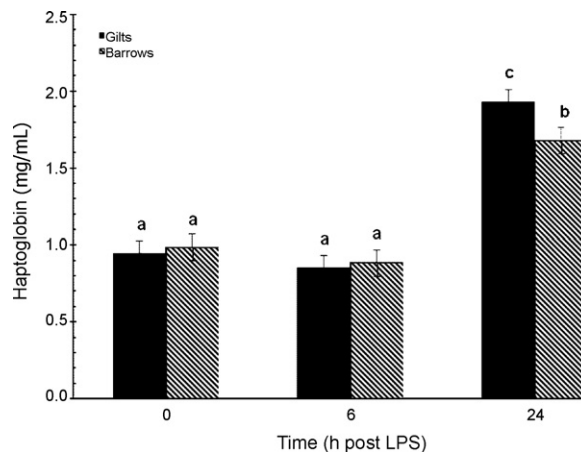


Fig. 7. Serum concentration of haptoglobin at 0, 6, and 24 h following an i.v. challenge with lipopolysaccharide (LPS;  $25 \mu\text{g/kg}$ ). Blood samples were taken at infusion (0 h) and then 6 and 24 h following LPS infusion. Values are represented as the mean  $\pm$  standard error of the mean ( $n = 26$ ). Lipopolysaccharide induced a time-dependent increase ( $P < 0.01$ ) in serum haptoglobin. Bars with different letters are different ( $P < 0.01$ ).

not affected by sex ( $P = 0.48$ ), and the temporal pattern of CRP response to LPS was not different between sexes ( $P = 0.97$ ). In contrast to CRP, serum haptoglobin concentration did not differ ( $P > 0.05$ ) from 0 to 6 h, and it subsequently doubled by 24 h post-LPS (Fig. 7). Although the main effect of sex was not a significant source of variation ( $P = 0.41$ ), gilts had a greater ( $P < 0.01$ ) concentration of haptoglobin at 24 h post-LPS ( $1.92 \pm 0.08$  vs  $1.68 \pm 0.08 \text{ mg/mL}$  for gilts and barrows, respectively). Similar to haptoglobin, SAA concentration was not different ( $P > 0.05$ ) from 0 to 6 h, but it then increased approximately 20-fold by 24 h post-LPS (Fig. 8). The response of SAA to LPS was influenced by sex, with barrows being more responsive ( $P < 0.05$ ), as reflected by an almost 70% greater ( $P < 0.01$ ) concentration of SAA in barrows as compared to gilts ( $195 \pm 28$  vs  $130 \pm 0.25 \text{ mg/mL}$ , respectively).

### 4. Discussion

Currently, little is known about the interactions between the SNS and the innate immune response following immune challenge. The present results support the hypothesis that challenge with LPS triggers a coordinated cascade of stress- and immune-related hormones, resulting in a repartitioning of immune cells. Another observation is that immune and stress reactions are influenced by sex. This appears to be the first study to report the temporal responses of catecholamines following an LPS challenge in pigs.

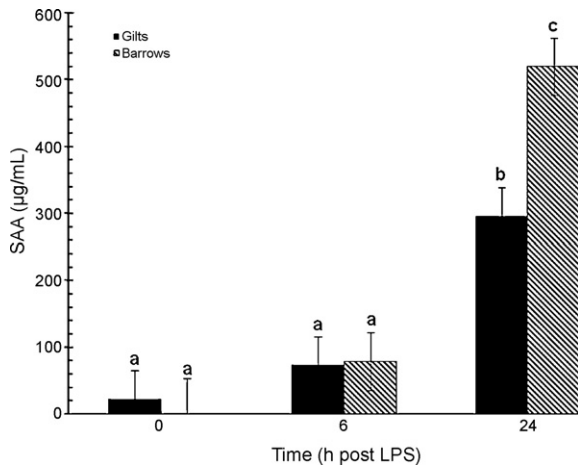


Fig. 8. Serum concentration of serum amyloid A (SAA) 0, 6, and 24 h following i.v. challenge with lipopolysaccharide (LPS; 25 µg/kg). Blood samples were taken for 0, 6, and 24 h following LPS infusion. Values are represented as the mean  $\pm$  standard error of the mean ( $n = 26$ ). Lipopolysaccharide induced a time-dependent increase ( $P < 0.01$ ) in serum SAA. There was a tendency for a main effect of sex ( $P = 0.09$ ), and a sex-by-time interaction was found ( $P < 0.05$ ). Barrows had an increased production of SAA compared to gilts at 24 h post-LPS. Bars with different letters are different ( $P < 0.01$ ).

The temporal pattern of TNF- $\alpha$  production following LPS found in this study is similar to those reported previously in the pig [11–13]. For example, Frank et al. [11] and Carroll et al. detected that peak TNF- $\alpha$  response occurred at 1 h post-LPS, with rapid decreases occurring after the peak [12,13]. In all previously cited reports, TNF- $\alpha$  concentration returned to baseline concentration between 3 and 8 h post-LPS. Similar to our data, Frank et al. [11] reported increases in IL-1 $\beta$  that were apparent by 1 to 2 h post-LPS. However, Frank et al. were unable to determine the time of peak, as IL-1 $\beta$  concentration had not yet begun to decrease by the end of the 4.5-h sampling period. Unlike previous reports [1,8,11] our data illustrate a biphasic IL-6 response following LPS, with peaks in production occurring at both 2.5 and 3.5 h post-LPS. This finding could be related to differences in sampling frequency, in that we sampled more frequently and over a longer period of time [1]. Although the biological significance of this biphasic pattern remains to be determined, a similar pattern has been observed in rats following LPS, with IL-6 peaking at 4 h and again at 16 h post-LPS, and may facilitate a sustained yet regulated immune reaction that prevents a hyperinflammatory response to antigenic challenge [14].

Sexually dimorphic immune system responses have been previously demonstrated in humans, mice, and rats [15,16]. However, this effect has not been extensively investigated in the immune response to LPS in pigs.

From the previous reports of pro-inflammatory cytokine response to LPS in pigs, only Webel et al. analyzed for the effect of sex and found it to be insignificant [1]. Other reports either included only barrows [3,11,12] or did not analyze for the effect of sex [8]. It has been suggested that sexually dimorphic immune responses may be due to the influence of sex steroids on the development and function of immune cells [15,16]. For example, incubation of mice macrophages with estradiol or progesterone before an endotoxin challenge decreased subsequent production of TNF- $\alpha$  and IL-6 [17,18]. Consistent with those reports, Imahara et al. [19] also demonstrated that compared to those in males, human whole blood cultures from females had decreased TNF- $\alpha$  and IL-1 $\beta$  production following exposure to LPS but did not differ in IL-6 production. Although the results of these studies are consistent with our data for IL-1 $\beta$  response, they are not consistent for TNF- $\alpha$  or IL-6 responses, in which it was the males that demonstrated a decreased response. Although the pigs used here are presumably too young to have pubertal levels of sex steroids, Ziecik et al. observed measurable concentrations of testosterone and estradiol in neonatal pigs [20], and in this study, intact, sexually immature gilts were compared to prepubertally castrated males. Therefore, the possible effect of sex hormones on differences in the amplitude of pro-inflammatory cytokine response to LPS shown here cannot be completely dismissed. However, as the present study used prepubertal animals, these data also suggest that there are inherent differences in the immune response of the sexes unrelated to the influence of sex steroids.

In general, these relationships are consistent with previous reports that pro-inflammatory cytokine production is capable of feeding back at the level of the brain to modulate activity of the HPA axis and promote the production of cortisol [2,6,21]. Likewise, the positive relationship between catecholamines and IL-6 production supports previous reports that catecholamines can induce the concentration of IL-6 in systemic circulation in the absence of antigenic challenge [4]. The present data also suggest that catecholamines may selectively enhance the production of other pro-inflammatory cytokines such as IL-1 $\beta$ .

Proliferation of leukocytes was readily apparent at 24 h post-LPS infusion, as indicated by an increase of approximately 55% in WBC relative to numbers at 0 h. Although there were increases in all WBC types, the largest quantitative increase occurred in the number of neutrophils. These results are consistent with the documented ability of the pro-inflammatory cytokines to either directly or indirectly, through the activation of Th cells, induce immune cell proliferation [22,23].

The role of the pro-inflammatory cytokines in inducing leukocyte proliferation is also supported by the positive relationships found between the magnitude of the TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 responses and total leukocyte counts and neutrophil counts at 24 h. There were also positive relationships found between the magnitude of the cortisol and E responses and total leukocyte and neutrophil counts at 24 h. Although these relationships may reflect only the previously described relationships existing between the magnitude of the pro-inflammatory response and stress hormone response, there is considerable *in vitro* and *in vivo* evidence that stress hormones can modulate the action of pro-inflammatory cytokines [21,24].

Consistent with the ability of pro-inflammatory cytokines to induce APPs [25,26], correlation analyses indicated a positive relationship between the magnitude of the IL-1 $\beta$  response and the concentration of SAA at 24 h post-LPS. There were also positive relationships found between the magnitude of the IL-6 response and the concentration of SAA and CRP at 24 h post-LPS. This latter relationship agrees with the documented ability of IL-6 to synergistically enhance the production of type 1 APP by IL-1 [27]. However, there was no significant relationship found between the magnitude of the TNF- $\alpha$  response and APP concentration at 24 h post-LPS, nor were there any relationships found between any of the pro-inflammatory cytokine responses and haptoglobin concentrations post-LPS. These results were unexpected, as haptoglobin is a type I APP, and both IL-1 $\beta$  and TNF- $\alpha$  induce type I APP production. Correlation analysis also indicated a strong positive relationship between the amplitude of the cortisol response and SAA response following LPS. Furthermore, the magnitude of the cortisol response along with the NE response were both positively related to SAA response. Cortisol has been reported to enhance the production of APP [26,27]. However, there is no previous evidence, to our knowledge, that relates the effects of catecholamines to APP production.

Although correlative data do not necessarily prove a direct role for stress hormones, the temporal patterns of stress hormones and pro-inflammatory cytokines following LPS are suggestive of a highly interactive role for these molecules in orchestrating leukocyte redistribution following LPS challenge. For example, the most immediate response to LPS challenge appears to be activation of the SNS, as reflected by substantial increases in circulating concentrations of NE and E. The negative relationships between these hormones and lymphocyte and monocyte numbers (quantitatively, the immune cells that account for the majority of the decrease in leukocyte

numbers) are suggestive of a direct role for these hormones in inducing the trafficking of these cell types out of the peripheral circulation. In support of this hypothesis, a number of reports have demonstrated the ability of acute stress (in the absence of antigenic challenge) or administration of catecholamines to induce a redistribution of leukocytes [21,24]. In the case of catecholamine administration, the redistribution event is characterized by a rapid mobilization of lymphocytes (ie, an increase in lymphocyte numbers within 30 min), followed by a dramatic decrease in lymphocyte numbers within 4 h [28,29]. Alternatively, the effects of NE and E could be mediated indirectly through the augmentation of IL-6 and IL-1 $\beta$  production.

Furthermore, the positive relationship found between the magnitude of the cortisol response and neutrophil numbers at 5.5 h post-LPS suggest that the HPA axis may also play a role in leukocyte redistribution by inhibiting the efflux of neutrophils from the peripheral circulation. In support of this hypothesis, it has also been found that the ability of acute stress (in the absence of antigenic stimulation) to increase the number of circulating neutrophils in mice is dependent on adrenal stress hormones [24].

Although other studies with pigs have not specifically reported the relationship between inflammatory cytokine production following LPS infusion and the stress response, the relationships reported in this study are consistent with the ability of LPS to cause activation and translocation of transcription factor NF- $\kappa$ B, which is known to induce the transcription of a wide array of pro-inflammatory cytokine genes [5,30,31]. Furthermore, the results presented here and elsewhere indicate sex-specific differences in immune and stress responses to antigenic challenges. Therefore, ideally, future studies would measure the expression of other cytokines not studied here, as the results may reveal new information on the pathways for system integration as it relates to immune and stress responses. Future studies could account for sex-based differences and provide insight into the reasons why sex influences the cumulative immune and stress responses to pathogenic challenge.

**Declaration of Interests:** The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work. Mention of trade names or proprietary products does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

**Funding:** This study was supported by: grant #5S06GM008107-33 from DHHS/NIH/NIGMS/MBRS to JCL; Texas AgriLife Research and USDA Animal

Health Formula funds to THW; and, USDA/CSREES Hispanic Serving Institution Grant Award #2005-38422-15945 to JCL and M. Garcia.

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